IDENTIFICATION, CHARACTERISATION AND TN5-MUTAGENESIS OF A FLUORESCENT PSEUDOMONAD ISOLATED IN NEW ZEALAND DISPLAYING STRONG ANTI-FUNGAL ACTIVITY

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ABSTRACT

Gilpin, B.J. & Palmer, B.R. (1992). Identification, characterisation and Tn5-mutagenesis of a fluorescent pseudomonad isolated in New Zealand displaying strong antifungal activity. *New Zealand Natural Sciences* 19: 87-94.

A fluorescent pseudomonad, PMS382, previously observed to inhibit fungal growth, was characterised and identified as *Pseudomonas aureofaciens*. Tn5-mutagenesis yielded 15 auxotrophic mutants and 4 mutants with reduced antibiosis against the fungus *Aphanomyces euteiches*. The latter mutants were identified using an *in vitro* bioassay system. The growth requirements of 12 of the auxotrophs were characterised.

Characterisation of the reduced antibiosis mutants indicates the importance of phenazine-1-carboxylic acid, cyanide and an additional unidentified anti-fungal agent in fungal inhibition.

KEYWORDS: *Pseudomonas aureofaciens* - anti-fungal activity - transposon mutagenesis - bacteria.

INTRODUCTION

Fluorescent pseudomonads are a group of bacteria which have shown potential for use in the protection of crops from fungal pathogens (Brisbane *et al.* 1989, Voisard *et al.* 1989, Thomashow *et al.* 1990). These bacteria are known to produce a wide variety of secondary metabolites, many of which have been shown to have antibiotic activity (Leisinger & Magraff 1979). In particular several isolates of *Pseudomonas fluorescens* have been shown to suppress phytopathogenic fungi both *in vitro* and in soil (Loper 1988, Brisbane *et al.* 1989, Voisard *et al.* 1989). Genetic studies indicate that genes involved in the production of hydrogen cyanide (HCN) and a variety of other inhibitory compounds are involved in this anti-fungal activity (Voisard *et al.* 1989, Keel *et al.* 1990).

Here we describe the identification, characterisation and mutagenesis of a bacterial isolate which has previously been shown to have strong anti-fungal activity. PMS382, a fluorescent pseudomonad, was isolated from mushroom compost and has been shown to strongly inhibit fungal growth when bioassayed against several phytopathogenic fungi (Rainey 1989, A.L.J. Cole, Dept. of Plant & Microbial Sciences, University of Canterbury, pers. comm.).

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MATERIALS AND METHODS

STRAINS

The bacterial strains used in this study are listed in Table 1. The fungus used in these experiments was *Aphanomyces euteiches* 6478, a New Zealand legume-pathogenic isolate held in the departmental collection originally obtained from F.R. Sanderson (Crop Research Division DSIR, Lincoln, New Zealand).

CULTURE MEDIA AND MEDIA SUPPLEMENTS

The complete media used were Potato Dextrose Agar (PDA) and Brain Heart Infusion (BH) from Difco Laboratories. The glucose based minimal medium (MM) of Davis & Mingioli (1950), the glycerol based minimal medium (GMM) of Salcher & Lingens (1980), King's B medium (KB) of King *et al.* (1954), and the Luria-Bertani medium (LB) of Sambrook *et al.* (1989) were used where appropriate. Where desired 1% Oxoid Agar No. 1 was added to solidify the media. The medium indicated as

Table 1. Bacterial strains used in this study

Designation	Characteristics[1]	Source or Reference
Pseudomonas aureofaciens		
PMS382	fungal inhibitor	Rainey (1989)
PMS382-40	Afa$^-$ Phe$^-$	This study
PMS382-41	antibiosis reduced	This study
PMS382-44	Afa$^-$ Phe$^-$ HCN-	This study
PMS382-45	antibiosis reduced, Phe$^-$	This study
Pseudomonas aeruginosa		
OT2	*leu-1* HCN$^+$	Loutit *et al.* (1968)
OT906	prototroph HCN$^-$	Lab stocks
Escherichia coli		
SM10	*thi thr leu* su_{III} integrated RP4-2-Tc::Mu (Km^r) pSUP2021 (Km^r Ap^r Cm^r Mob$^+$)	Simon *et al.* (1983)

[1]Afa (anti-fungal activity at a distance), Phe (phenazine production), HCN (hydrogen cyanide production).

½PDA/BH was half strength PDA and BH in equal quantities with a total concentration of 1% agar. King's B medium was supplemented as required with 200 μg ml^{-1} ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA).

Antibiotics were added to sterile media at the following concentrations: ampicillin (Ap) 50 μg ml^{-1}, streptomycin (Sm) 250 μg ml^{-1}, kanamycin (Km) 100 μg ml^{-1}.

Stationary phase BH broth cultures of *Pseudomonas* species were supplemented with 15% sterile glycerol and stored at -80°C. *E.coli* stock cultures were stored in 40% sterile glycerol at -80°C.

Enzymes and biochemicals

*Eco*RI was obtained from Boehringer Mannheim and the DNA nick translation kit was obtained from Amersham International.

All chemicals used were of analytical grade. Phenazine-1-carboxylic acid (PCA) was supplied by P. Brisbane (CSIRO Division of Soils, Glen Osmond, South Australia). The nucleotide used for radioisotopic labelling was 5'-[^{32}P]-dCTP triethylammonium salt obtained from Amersham International.

Identification of PMS382

Gram-staining, catalase, oxidase, and motility tests were performed as described in Gerhardt *et al.* (1981). Known positive and negative controls were used where possible. The growth of PMS382 on various substrates was analysed using a API 20NE strip as per the manufacturer's instructions (API Systems, France) and incubated at 30°C.

For examination using transmission electron microscopy (TEM), bacterial colonies were emulsified in glass distilled water and stained with 2% phosphotungstic acid before examination using a TEOL TEM 1200EX.

Antibiotic resistance

Lawns of PMS382 on BH plates were tested for antibiotic resistance using sterile paper discs (1 cm diameter) containing aliquots of antibiotic stock solutions. *P. aeruginosa* strain OT2, which is sensitive to all the antibiotics used, was used as a control. Antibiotics tested were: kanamycin (Km), chloramphenicol (Cm), tetracycline (Tc), carbenicillin (Cb), streptomycin (Sm) and gentamicin (Gm). Plates were incubated at 37°C for 24 h before comparing zones of inhibition.

Cyanide evolution

Cyanide production was detected using an alkaline picric acid test (Michaels & Corpe 1965). A saturated solution of picric acid (2,4,6-trinitrophe-

nol) was adjusted to pH 7.0 with anhydrous $NaHCO_3$. Filter paper discs were soaked in this solution and then dried in a 55°C oven. The discs were stored in an airtight container at room temperature. Since HCN has a boiling point of 26°C and is less dense than air, HCN produced will be given off as a gas. Appropriately sized discs, placed in the lid of a universal or bijou bottle, change colour from yellow to orange when cyanide is produced by a bacterial culture.

ISOLATION OF TRANSPOSON-INSERTION MUTANTS

Tn5-mediated mutagenesis was carried out as described by Marugg *et al.* (1985) using the donor strain *E.coli* SM10, harbouring pSUP2021, and PMS382 which were both grown to exponential phase in BH. Aliquots (100 µl) of the final mating mixture were spread on BH plates supplemented with Km and Sm and incubated for 36 h at 37°C. Putative Tn5-insertion mutants were retested for resistance to Km and Sm and strongly resistant clones subjected to screens for auxotrophy, loss of anti-fungal activity, fluorescence and cyanide production.

GROWTH REQUIREMENTS OF AUXOTROPHIC MUTANTS

Single colonies were transferred by toothpick onto BH and MM plates and incubated overnight at 30°C. Colonies growing on BH but not MM, were retested and designated auxotrophic. These auxotrophic mutants were tested for dependence on 39 growth factors using the method of auxanography adapted from Holliday (1956) and Davis *et al.* (1980). All biochemical requirements were confirmed by repeating the test with only a single growth factor in a well, and finally on MA plates supplemented with the appropriate biochemical.

FLUORESCENCE

Bacterial colonies were tested for fluorescence after growth on Kings B medium or BH plates under UV illumination.

ANTIBIOSIS

A 5 mm plug of *A. euteiches* 6478 from a colony on PDA was placed in the centre of a ½PDA/BH plate and 10 to 12 Tn5-generated mutants and the wild type PMS382 were inoculated around the edge of the plate. Plates were incubated for 3-4 days and mutants with a reduced inhibition zone as compared to PMS382 were selected for further study. For more rigourous screening of anti-fungal activity PMS382, SM10 and each mutant were streaked equidistantly from a plug of *A. euteiches* 6478, incubated at 30°C and examined daily.

THIN LAYER CHROMATOGRAPHY

Liquid cultures of *P. aureofaciens* were examined using thin layer chromatography (TLC) for the production of phenazine-like compounds as described by (Salcher & Lingens 1980). Each strain was grown for five days at 30°C in an orbital shaker in 10 ml of the standard glucose based MM media and GMM. Each sample was extracted once with ethyl acetate. The organic layer was separated and concentrated *in vacuo*. The samples were applied to Schleicher & Schuell G 1500/LS 254 silicagel + indicator and co-chromatographed with PCA and anthranilic acid. The TLC was run for approximately 10 min using ethyl acetate/propan-2-ol/water (65:24:11, by vol) as the solvent. Plates were examined under both short and long wave ultraviolet light.

DNA EXTRACTION AND ANALYSIS

Chromosomal DNA was extracted using the method of Owen & Borman (1987). pSUP2021 DNA was prepared via the rapid boiling method of Holmes & Quigley (1981). Restriction endonuclease digestions were performed as detailed by the manufacturers. Agarose gel electrophoresis was performed using 0.7% or 1% (w/v) standard agarose (Bio-Rad Laboratories) dissolved in TBE buffer as described in Sambrook *et al.* (1989).

Southern transfers of agarose gels and subsequent hybridisation procedures were performed according to the methods of Meinkoth & Wahl (1984) using a BA45 nitrocellulose membrane filter (Schleicher & Schuell). pSUP2021 plasmid DNA and BRL 1 kb ladder DNA probes were labelled with ^{32}P-dCTP by nick translation.

RESULTS

IDENTIFICATION OF PMS382

In an effort to identify PMS382 to species level, the bacterium was subjected to the various biochemical and morphological tests described. Table 2 outlines the main characteristics used in the iden-

Table 2. Morphological and biochemical characteristics of PMS382.

Test or characteristic		Result[1]
Gram stain reaction		-ve
Oxidase test		+ve
Catalase test		+ve
Arginine dihydrolase		+ve
Motility test		+ve
Morphology		rod
Cell size		0.5 μm wide by 1.0 to 1.8 μm long
Growth at:	4°C	+ve
	37°C	+ve
	42°C	-ve
Antibiotic resistance:		
Kanamycin		S (50 μg ml^{-1})
Chloramphenicol		S (50 μg ml^{-1})
Tetracycline		R (50 μg ml^{-1})
Carbenicillin		R (150 μg ml^{-1})
Streptomycin		R (300 μg ml^{-1})
Gentamycin		R (10 μg ml^{-1})

[1]S = sensitive, R = resistant (indicates a zone of inhibition < 3 mm).

tification of PMS382. On KB media PMS382 fluoresces yellow/green and on KB, BH, PDA, NA, and MM, it produces an orange pigment which diffuses into the media. TEM revealed the presence of three to five polar flagella.

PMS382 has an API 20NE profile of 1157557 which keys out as *Pseudomonas aureofaciens* with a 93.1% probability (T=0.97). The Gram-reaction, fluorescence, pigment production and the number and position of flagella were also indicative of *P. aureofaciens*. This identification was confirmed by examination of the keys in Bergeys Manual of Systematic Bacteriology (Kreig & Holt 1984).

Cyanide production

As many strains of *P. aureofaciens* produce HCN, PMS382 was examined for possible HCN production using alkaline picric acid discs as described. The picric acid discs showed an intense colour change from yellow to a deep orange/brown. This indicated PMS382 produces considerable amounts of HCN as compared to *P. aeruginosa* OT2 which exhibited moderate orange colour formation (HCN production) using this test. HCN production by PMS382 was temperature dependent as it was not produced at 34°C or above. From the intensity of the colour change of the discs, more HCN was produced at 30°C than 32°C. The negative controls, *E. coli* SM10 and *P. aeruginosa* OT906, caused no change in the colour of the discs at any temperature tested.

Tn5-mutagenesis of PMS382

An antibiogram of PMS382 revealed this organism to be sensitive to Km and Cm and resistant to Tc, Cb, Sm and Gm at the levels tested (Table 2). From this information, Tn5-mutagenesis was deemed appropriate for the generation of mutants of PMS382 with reduced anti-fungal activity. Several thousand Km and Sm resistant (Km^R and Sm^R) mutants were generated using the mutagenesis protocol outlined above. Clearly separated colonies were screened for auxotrophy and loss of antibiosis.

Auxotrophy

As an indicator of the efficiency of Tn5 insertion the frequency of auxotrophic mutations, arising from Tn5-mutagenesis was determined by screening colonies for loss of prototrophy. Fifteen auxotrophic mutants were found amongst the 2137 colonies screened (mutation rate of 0.7%). Their growth requirements were characterised on minimal agar using auxanography. Four mutants required arginine, three tryptophan, two histidine, one glycine, one methionine, cysteine or cystine, one methionine or cystine and three were not able to be characterised using the conditions employed.

Antibiosis

Nine hundred and eighty-four putative Tn5 mutants were screened for loss of anti-fungal activity against *A. euteiches*. *A. euteiches* was chosen as the test fungus as it is a plant pathogen, particularly of legumes, is strongly inhibited by PMS382 and will grow on common laboratory media relatively quickly at 30°C. The medium ½PDA/BH agar was employed in the bioassays rather than PDA and BH alone in order to achieve optimum growth of both bacterium and fungus. After rescreening four mutants which displayed consistently reduced-antibiosis were identified (mutation rate 0.4%).

To reduce problems caused by plate-to-plate

variability, each mutant was tested for its ability to inhibit *A. euteiches* on a single plate and PMS382 and SM10 were included as positive and negative controls, respectively. Mutants PMS382-41 and PMS382-45 displayed reduced antibiosis against *A. euteiches* (Table 3), and mutants PMS382-40 and PMS382-44 did not produce inhibition zones at all, allowing the fungus to grow up to the edges of the bacterial colonies. However, some anti-fungal activity was retained, the bacterial cells of these two mutants appearing to colonise the edge of the fungal mycelium causing hyphal deterioration and death.

THIN LAYER CHROMOTOGRAPHY

Those mutants which failed to produce the orange PCA pigment (Table 3) were compared with PMS382 for production of phenazine-associated pigments by TLC. Table 4 shows the results of the TLC of extracts from PMS382, PMS382-44 and PMS382-45. Examination of the chromatograph of the glucose based minimal media extracts revealed that the PMS382 extract fractionated into two bands with R_f values of 0.94 and 0.89, the latter corresponding to the PCA standard. Mutant PMS382-44 had a very faint spot at R_f 0.62, while mutant PMS382-45 showed two very faint spots corresponding to those of PMS382 and a third at R_f 0.96.

The extracts from the cultures grown in GMM showed a number of differences from the chromatographs produced from cultures grown in glucose MM. The PMS382 extract again separated into two bands. The extract from mutant PMS382-45 appeared to be a composite of PMS382 and PMS382-45 from the glucose MM with spots at R_f 0.88, 0.94 and 0.97. In addition it had a number of spots at less than R_f 0.45. Examination of these TLC plates under long wavelength UV (data not shown) revealed the same pattern of spots but the PCA spot was more clearly fluorescent when absorbing at this wavelength than under short wavelength UV.

TRANSPOSON COPY NUMBER

*Eco*RI-digested DNA from PMS382 and its four anti-fungal mutant derivatives was subjected to Southern hybridisation using a pSUP2021 ^{32}P-labelled probe. A single DNA fragment from each of the mutants hybridised to the probe suggesting each mutant chromosome contained only a single Tn5 insertion (data not shown).

DISCUSSION

The novel New Zealand bacterial isolate PMS382 has been identified as *P. aureofaciens* using both biochemical and morphological characteristics. Fatty acid analysis performed on this strain is also indicative of PMS382 being *P. aureofaciens* although the fatty acid profile suggests it is a distinct isolate differing from existing library entries (Rainey, pers. comm.). *P. aureofaciens* has been shown to produce a number of compounds which have anti-fungal activity (Trutko *et al.* 1989, Vincent *et al.* 1991) with a few strains producing HCN (Knowles & Bunch 1986). Among bacteria, only a few species in the genera *Chromobacterium* and *Pseudomonas* produce HCN (Castric 1977). Cyanogenesis has been observed in about 70% of *P. aeruginosa* strains (Castric 1981), many strains of *P. fluorescens* and some isolates of *P. chlororaphis* (Knowles & Bunch 1986). The distinctive deep

Table 3. Characteristics of selected mutants.

Mutant	Antibiosis[1]	Fluorescence[2] on Kings B	Cyanide Production	Phenazine Production	Number of Tn5 inserts
PMS382	+	+	+	+	none
PMS382-40	-	+	+	-	one
PMS382-41	reduced	+	reduced	reduced	one
PMS382-44	-	+	-	-	one
PMS382-45	reduced	+	+	-	one

[1]As determined by production of a zone of inhibition with *A. euteiches*.

[2]Growth of all strains was unaffected by the addition of the iron chelator EDDA to Kings B plates which has been shown to inhibit the growth of fluorescent pseudomonads deficient in the production of siderophores (Rombel & Lamont, 1992).

Table 4. Detection of inhibitory compounds produced by PMS382 and derivatives using TLC.

Compound:	Anthranilic acid		PCA	
Strain	MM[1]	GMM[1]	MM[1]	GMM[1]
PMS382	++	++	++	++
PMS382-44	-	-	-	+/-
PMS382-45	+/-	++	+/-	++

[1]Growth medium used.

orange colour of *P. aureofaciens* colonies is attributed to the presence of phenazines within the cells (Olson & Richards 1967, Turner & Messenger, 1986).

PMS382 shows strong anti-fungal activity and Rainey (1989) compares its inhibition of *Agaricus bisporus* mycelium to that of the virulent mushroom pathogen *P. tolaasii*. PMS382 was shown in this study to produce HCN and PCA. There is mounting evidence that the production of HCN (Voisard *et al.* 1989) and phenazines (Thomashow *et al.* 1990) by rhizosphere bacteria play a role in the suppression of plant diseases caused by fungi.

In an effort to elucidate whether other compounds besides HCN and phenazines might be involved in the anti-fungal activity of *P. aureofaciens* PMS382, it was subjected to Tn5-mutagenesis. PMS382 proved readily amenable to transposon mutagenesis with several auxotrophic mutants and four mutants with reduced anti-fungal activity being isolated following *in vitro* bioassays against *A. euteiches*, a fungal pathogen of legumes. In contrast to screening for the loss of anti-fungal agent production (Voisard *et al.* 1989, Vincent *et al.* 1991) this bioassay system should allow the isolation of mutants deficient in the production of any inhibitory compound produced under these conditions. These mutants should prove useful in the further study of the anti-fungal activity exhibited by PMS382. The auxotrophic mutants are a suitable resource from which to build up genomic mapping data for *P. aureofaciens*.

The phenotypes of these anti-fungal-deficient mutants indicate that while HCN and phenazine production are involved in anti-fungal activity at least one other anti-fungal compound is produced by PMS382. This is indicated by the fact that all four anti-fungal mutants retain some anti-fungal activity against *A. euteiches* as all can at least inhibit the fungus upon contact. Two mutants PMS382-40 and PMS382-44 require physical contact between the bacterial colony and fungal hyphae to exhibit anti-fungal activity and one of these, PMS382-44, was shown to produce neither HCN nor phenazine. Neither PMS382-40 nor PMS382-44 produces PCA, suggesting a role for phenazine in forming a zone of inhibition. Why PMS382-45 (PCA^-) still produces an inhibition zone is unknown, but a possible explanation is its ability to form PCA and other related metabolites under certain growth conditions. While cyanogenesis does not appear essential to inhibit *A. euteiches* without direct contact synergistic effects were not tested rigourously (Table 3).

One possible group of candidate compounds with potential anti-fungal activity are the fluorescent siderophore molecules which efficiently chelate ferric ions (Leong 1986). Siderophores are commonly produced by pseudomonads of the *P. fluorescens* complex (Leisinger & Magraff 1979) and some studies indicate that an excess of ferric ions in the growth medium reduces fungal inhibition by bacteria (Kloepper *et al.* 1980, Becker & Cook 1988). However, other groups question the relative importance of siderophores in disease suppression (Brisbane *et al.* 1989, Flaishman *et al.* 1990). All the PMS382 mutants with reduced antibiosis discussed here produced fluorescent pigments. Another compound with inhibitory activity towards fungi which is produced by some strains of *P. aureofaciens* is 2,4-diacetylphloroglucinol (Phl) (Vincent *et al.* 1991). Mutagenised PMS382 were not screened for the loss of production of Phl in this study.

The examination of mutants PMS382-44 and PMS382-45 for phenazine production by TLC of cell extracts showed that both mutants produced greatly reduced amounts of PCA and related compounds when grown with glucose as the carbon source. PMS382-45 produced a range of compounds which was virtually identical to those produced by PMS382 when glycerol was the carbon source. This suggests that PMS382-45 may carry a regulatory mutation of some sort that alters the wild type metabolic response to glucose as a substrate. The mutant PMS382-44 has an interesting phenotype in that it is blocked in production of both HCN and PCA. Since Southern hybridisation to DNA

from all four anti-fungal-deficient mutants revealed each contains a single Tn5 insert, this phenotype is presumably the result of mutation of a single gene. This may represent a mutation in either a regulatory gene, having effects on both HCN- and phenazine-production pathways, or a gene encoding an enzyme common to both pathways.

It has been shown that PMS382 can associate with plant roots (Rainey, pers. comm.). Studies are now required to investigate whether PMS382 can protect plants from fungal attack and whether mutants deficient in fungal inhibitory activity *in vitro* are also deficient in the rhizosphere. Work aimed at identifying the mutated genes in the mutants deficient in anti-fungal activity is continuing.

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